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The origin of the stapes and relationship to the otic capsule and oval window

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Key Findings:

- The stapedial footplate is of neural crest and mesoderm origin.
- The otic capsule contributes to the stapedial footplate.
- The neural crest component of the stapedial footplate is needed for development of the mesodermal component and for proper formation of the oval window.
- The position of the oval window appears independent of the stapes

Abstract:

Background: The stapes, an ossicle found within the middle ear, is involved in transmitting sound waves to the inner ear via the oval window. There are a number of developmental problems associated with this ossicle and the oval window, including stapes fixation or defects in the oval window, which cause hearing loss. The developmental origin of these tissues has not been fully elucidated.

Results: Using transgenic reporter mice we have shown that the stapes is of dual origin with the stapedial footplate being composed of cells of both neural crest and mesodermal origin. *Wnt1cre/Dicer* mice fail to develop neural crest cartilages, therefore have no middle ear ossicles. We have shown in these mice the mesodermal stapedial footplate fails to form and the oval window is induced but under developed.

Conclusion: If the neural crest part of the stapes fails to form the mesodermal part does not develop, indicating that the two parts are interdependent. The stapes develops tightly associated with the otic capsule, however it is not essential for the positioning of the oval window, suggesting that other tissues, perhaps within the inner ear are needed for oval window placement.

Introduction

Deafness and hearing loss affects 275 million people in the world (WHO estimate 2004), and congenital hearing loss, which is due to a developmental defect, affects about 1 in 1000 live births (NHS screening committee, American Hearing research foundation). This includes problems with the development of the middle ear ossicles (malleus, incus and stapes), of which stapes anomalies are the most frequent (Park et al., 2009). The stapes is the last ossicle in the chain and is involved with transmitting sound waves from the incus (central ossicle in the chain) to the inner ear (Figure 1A). Stapes defects include stapes displacement, stapes fixation (ankylosis) and stapes dysplasia. Of these anomalies, stapedial footplate fixation is the most common and generally affects both ears (Park et al., 2009). Without a correctly functioning stapes, sound waves are unable to be transmitted to the inner ear, and hearing is impaired.

The stapes is a stirrup-shaped structure with its head connected to the incus via the incudostapedial joint; its two arms (crus) surround the stapedial foramen and join at the base to form the stapedial footplate (Figure 1A,B). The stapedial footplate is inserted in the oval window (fenestra ovalis) of the otic capsule, abutting the oval window membrane and attached to the otic capsule by the annular ligament. During development the stapedial artery passes through the stapedial foramen (Figure 1A), however this later degenerates in most mammals.

Cranial neural crest cells migrate out of the dorsal neural tube filling the pharyngeal arches. The first arch crest will form the upper and lower jaw bones along with the body of the malleus and incus of the mammalian middle ear, while the stapes and hyoid bones form from the second arch crest (O'Gorman, 2005). The origin of the stapedial footplate has been questioned for over a hundred years (Smith, 1904), mostly based on analysis of the avian columella- the homologous structure to the mammalian stapes and the only middle ear ossicles in non-mammalian terrestrial vertebrates. Histology and quail graft fate mapping techniques have revealed conflicting results with some groups suggesting that neural crest cells contribute to the whole of the avian columella (Le Lièvre, 1978; Couly et al., 1993), while Smith (1904) and more recently Noden (1986) suggest the columella to be of neural crest and mesoderm origin. A dual origin was also suggested by Kontges and Lumsden (1996), since the footplate was not populated by grafted quail neural crest.

Although the chick-quail technique has been used successfully to trace neural crest cells in many structures (Le Lièvre and Le Douarin, 1975), the results are limited by the accuracy of the grafting and the time when the graft is performed, resulting in some variability. In humans, some histological studies have indicated that the stapes footplate is derived from the otic capsule (Cauldwell and Anson, 1942; Bast and Anson, 1949; Hanson et al., 1962; Masuda et al., 1978), while later studies report that the stapes has no mesoderm derivatives and develops independently from the otic capsule (Rodríguez-Vázquez, 2005). In mice retinoic acid treatment has been shown to lead to formation of a complete stapes, at a distance from the otic capsule, indicating again an independent origin (Louryan et al., 2003). More recently, using a transgenic approach utilising *Hoxb1cre* mice, it has been shown that the stapes is composed of rhombomere 4 (r4)/ second-arch neural crest along with cells of another origin (O'Gorman, 2005); however this study does not ascertain the origin of these cells. As this cre line only labels r4 crest the negative cells in the stapes could be formed of crest cells from another rhombomere or originate from a non-neural crest population of cells.

Ablation of the otic capsule in the chick, turtle and urodele leads to a defect in the footplate and shaft of the columella (Reagan, 1917; Toerien, 1963; Toerien, 1965; Zou et al., 2012). Although this indicates that the footplate of the columella might be derived from the otic capsule, it has also been interpreted as the otic capsule being the source of signals that induce the formation of a neural crest derived footplate, without contribution from the mesoderm itself (Toerien, 1963; Toerien, 1965). It is therefore difficult to ascertain cell origin from such ablation experiments.

The annular ligament forms around the stapedial footplate holding it within the oval window. The development of this structure appears coordinated by the stapedial footplate and surrounding otic capsule. In humans, defects in the stapes' are highly correlated with absence of the oval window (Lambert, 1990; Zeifer et al., 2000). From a study of 51 ears with a missing oval window, only 2 showed a normal stapes (Jahrsdoerfer, 1977). The presence of an oval window therefore appears dependent on induction by signals from the stapes. A neural crest origin of the stapes places the forming oval window and annular ligament at the interface between the neural crest and mesodermal tissue. Signaling at the boundary of tissues of two origins is well known (Meinhardt, 1983), for example, the boundary between the mesoderm-derived parietal bones and cranial neural crest-derived frontal bone forms

the coronal suture (Jiang et al., 2002). If the signaling pathways are altered in the cranial sutures this can lead to craniosynostosis (Reviewed by Morriss-Kay and Wilkie, 2005), if this occurs in the developing annular ligament it could lead to stapes fixation and conductive hearing loss.

The origin of the cells within the stapes, in particular the footplate, is therefore still a point of contention. Elucidating this will give us a better understanding of the formation of the stapes and oval window and help provide an explanation for why and how stapes fixation occurs. Using cre/lox transgenic technology in mice, embryonic cells can now be permanently labeled and their fate determined. In this study, *Wnt1cre* and *Mesp1cre* mice were crossed with the reporter mouse R26R, to produce offspring expressing β-galactosidase in cells of neural crest or mesoderm origin respectively. This technology was utilised to investigate the origin of the stapedial footplate, while knockout mice were analysed to investigate the relationship of the stapes to the oval window.

Results

The development of the mouse stapedial footplate

At E14.5 the centre of the stapes and the otic capsule at a slight distance from the forming oval window stained positive for the cartilage marker Alcian Blue (Figure 1C). The footplate of the stapes could be distinguished as a structure distinct from the surrounding otic capsule. At E16.5, Alcian Blue staining strongly labeled chondrocytes within most of the stapes, except a halo around the footplate (arrow in Figure 1D). The footplate of the stapes was separated from the cartilaginous otic capsule by a dense region of Alcian Blue negative tissue (Figure 1D, arrow heads) - corresponding to the developing oval window. By E18.5, the stapes contained some hypertrophic chondroctyes and appeared to be surrounded by a forming perichondrium. The oval window had formed and the position of the developing annular ligament was clear (black arrow heads, Figure 1E). A region of less developed cartilage without hypertrophic chondrocytes, was visible adjacent to the annular ligament (Figure 1E, black arrows). During postnatal development the stapes undergoes endochondral ossification (Lyell, 1926). At postnatal (P) 6, the initiation of ossification was observed by staining with Sirius Red (Figure 1F). The annular ligament (arrow heads) was clearly distinguished holding the footplate in the oval window, while the cartilage attached to the annular ligament was still distinct from the main body of the stapes.

The footplate of the stapes is of dual origin

The stapes is composed of a head and a footplate connected by two rods called crus, which surround the foramen (Figure 1B). At P6 the whole of the stapes (crus, head, footplate) stained blue with the cartilage marker Alcian Blue (Figure 2A). In order to determine the embryonic origin of this structure, we used transgenic *Wnt1cre* and *Mesp1cre* mice crossed with the R26R reporter line. The *Wnt1cre*/R26R mouse labels neural crest derived tissue, while the *Mesp1cre*/R26R mouse labels cells of mesoderm origin (Danielian et al., 1998; Saga et al., 1999; Chai et al., 2000). At P10 neural crest derived tissue was shown to form the crus, head and base of the footplate of the stapes, however, the outer rim of the footplate was derived from *Wnt1cre*-negative cells (Figure 2B). Conversely, the outer rim of the stapedial footplate was stained blue in the *Mesp1cre*/R26R mouse, while the rest of the stapedial footplate was unstained (Figure 2C). To assess the border more accurately, the cre lines were

sectioned across the developing oval window (Figure 1D) and stained with X-gal. The crus and centre of the footplate were clearly neural crest derived (blue stain in Figure 2E), however the outer rim of the footplate, the annular ligament and otic capsule were mesoderm derived (blue stain in Figure 2F). The dual origin of the stapes is therefore confirmed, and the developing oval window forms within the mesoderm and not at the border between the crest and mesoderm derived tissues.

It has previously been reported that the otic capsule, like the stapes, is of dual origin in birds and mammals (Le Lièvre, 1978; O'Gorman, 2005). To identify the extent of neural crest contribution to the otic capsule we looked for stained cells in the *Wnt1cre*/R26R otic capsule. We found only a small region of the otic capsule was composed of neural crest cells (Figure 2G, H). This small region of the otic capsule sits above the malleus and incus and extends towards the round window, and is part of the pars canalicularis, which encases the semicircular canals. The stapes, incus and Reichert's cartilage (which goes on to form the styloid process) can also be seen in blue as they are known to be derived from neural crest (O'Gorman, 2005) (Figure 2G). The embryonic origin of the perichondrium of the stapes and otic capsule appears to correlate with the embryonic origin of the cartilage it covers (Figure 2E, F, H).

The relationship of the stapes to the otic capsule

For the columella, it has been suggested that this ossicle forms at a distance from the otic capsule and then inserts into the capsule as it develops (Jaskoll & Maderson, 1978). In order to determine the early relationship between the stapes and otic capsule in the mouse we used Sox9 immunohistochemistry to label the condensing pre-cartilaginous mesenchyme, and *Wnt1cre* mice to identify the origin of these cartilaginous cells. The stapes can be distinguished from the other ossicles during early development by its close proximity to the facial nerve and stapedial artery (Figure 1A). At E12.5 the neural crest derived (*Wnt1cre* positive) part of the stapedial footplate could be seen protruding into a neural crest negative region at E12.5 (arrow in Figure 3A). At this stage Sox9 was expressed in the developing chondrocytes of the stapes head, footplate and otic capsule, in addition to staining the vestibule of the inner ear (Figure 3A'). When these images were combined it is clear the neural crest derived footplate was already embedded in the otic capsule at this

early stage and surrounded by the Sox9 positive non-neural crest derived tissue which will form the mesodermal part of the footplate and otic capsule (Figure 3A").

A similar relationship of the stapes and otic capsule was observed at E15.5, with the neural crest cells bulging into a neural crest negative region (Figure 3B). However, at this stage the Sox9 immuno-staining showed a constriction in the forming otic capsule adjacent to the stapedial footplate (Figure 3E'); indicating the developing annular ligament. In combination, it could clearly be seen that the stapes was composed of neural crest and non-neural crest tissue (Figure 3E"), with the non-neural crest component abutting the oval window membrane and annular ligament.

The neural crest derived footplate is needed for normal development of the mesodermal footplate and oval window

Given the dual origin of the stapes and the possible role of the stapes in induction of the oval window we wanted to investigate the effect of loss of the neural crest part of the stapes on development of the mesodermal component of the stapes and the oval window. For this we took advantage of the *Wnt1cre*/Dicer mouse, which has been shown to lack cartilages of neural crest origin, including the stapes (Huang et al., 2010). In these mutants, migration of crest into the head appears normal but the cells do not differentiate into cartilage and high apoptosis is observed in the mandible mesenchyme at E12.5 (Huang et al., 2010; Zehir et al., 2010). At E18.5, in the wildtype mouse the middle ear ossicles can be seen strongly stained with Alcian Blue (Figure 4A). This was compared to the *Wnt1cre*/Dicer mouse, where we confirmed that all neural crest-derived cartilages were absent from the middle ear including the stapes (Figure 4B). If the neural crest part of the stapes interacts with the mesodermal part then specific loss of the neural crest derived tissue might result in a defect in the mesodermal footplate. Alternatively, if the two parts develop in isolation then loss of the neural crest might result in the mesodermal portion of the footplate being suspended within the oval window surrounded by annular ligament (Figure 4C, D). In wildtype mice the stapes can be seen positioned in the oval window held in place by the annular ligament at E18.5 (Figure 4C, E). In contrast, in the *Wnt1cre/Dicer* mutants a break in the otic capsule in the position of the presumptive oval window was observed at E18.5, despite the lack of neural crest derived stapes (Figure 4D', F). This diminutive "oval window" looked similar to the forming annular ligament seen in the wildtype (Figure 4E, F), and did not contain any cartilage structure. A similar

rudimentary oval window was observed at E14.5 and E16.5 in the mutants. The mesodermal part of the stapedial footplate, therefore appears not to form in the absence of the main body of the stapes. The neural crest derived part of the otic capsule was also missing in the *Wnt1cre/*Dicer mutants (Figure 4G, H).

Discussion

The origin of the footplate of the stapes, and its homologous structure in non-mammalian vertebrates the columella footplate, has to date been controversial. Studies by Noden (Noden, 1986; Noden & Van de Water, 1986) using the avian columella, provided evidence that the columella was of neural crest and mesoderm origin, while others showed it to be completely neural crest cells derived (Le Lièvre, 1978; Couly et al., 1993). More recently Kontges and Lumsden (1996) showed the columella to be of mainly neural crest origin with a small number of non-neural crest cells present. However, they did not determine the origin of these cells. In the mouse, lineage studies have also suggested a mixed origin of the stapes (O'Gorman, 2005). We have confirmed the hypothesis postulated by Bast & Anson (1949) that the mammalian stapes is composed of not only neural crest cells but also of mesodermal cells. The head and crus of the stapes is of neural crest origin, along with the central part of the stapedial footplate, however the outer ring of the stapedial footplate is of mesoderm origin. This is the part of the stapes that connects to the mesodermal annular ligament. Part of the stapes is therefore derived from the otic capsule.

The stapedial footplate is a composite structure

Evolution of vertebrates along with the emergence of the neural crest has lead to the development of complex sense organs, including the ear. The stapes or columella are found in all terrestrial vertebrates connecting the inner and outer ears. In the most primitive known tetrapod, the extinct *Acanthostega*, the stapedial footplate is firmly attached to the vestibular wall allowing no free movement of this ancient structure (Clack, 1989). The development of the annular ligament giving movement to the stapes would therefore have been the next step in auditory evolution and according to our results segregated part of the mesodermal otic vesicle into the stapedial footplate.

Most of the bones in the head are of a single origin either mesoderm or neural crest, making the stapes' dual origin an interesting finding. This however, is not a unique feature of the stapes as other bones of mixed origin include; the otic capsule (Le Lièvre, 1978), the sphenoid (Couly et al., 1993) and the interparietal bone (Jiang et al., 2002). The cells within the stapes retain a strict boundary demarcating the neural crest and mesoderm cells, with limited or no mixing of cells of different origin.

The stapedial footplate and otic capsule form together

The footplate of the stapes forms adjacent to the developing inner ear vestibule, with the otic capsule forming around it. The stapes therefore does not become associated with the otic capsule as it develops but is intricately linked with it from the start of development, at odds with the previous descriptions of the columella footplate in the chick (Jaskoll and Maderson, 1978). We have shown the stapes and the otic capsule develop concurrently, with mesenchyme condensation occurring at E12.5, shown by the simultaneous expression of the pre-chondrogenic marker- Sox9. Expression of the cartilage extracellular matrix glycoaminoglycans that stain blue with Alcian Blue can be seen in both the otic capsule and stapes by E16.5. This demonstrates the development of these two structures occurs at the same time and it is likely that this process is highly regulated to coordinate accurate growth and interaction. In keeping with this, a recent paper has shown the coordinated induction of the otic capsule and columella in the chick, specified by signals from the paraxial mesoderm (Zou et al., 2012). In mammals, chondrogenesis of the mesenchymal otic capsule is induced by the epithelial otic vesicle (McPhee and Van de Water, 1986) in a classic epithelial-mesenchymal signaling mechanism. Signaling molecules from the otic vesicle include TGFb (Frenz et al., 1992), FGF2 (Frenz et al., 1994), BMP4 (Liu et al., 2003) and Shh (Liu et al., 2002). The signaling pathways involved with stapes development are not yet known and it would be interesting to determine if the molecules involved with otic capsule induction also control stapedial footplate formation.

Although our studies were performed in the mouse it is likely that the avian columella shares this dual origin, with a mesodermal footplate, agreeing with the work of Noden (Noden, 1986; Noden & Van de Water, 1986). Study of osteogenesis of periosteum cells in the columella has shown two initiation sites, an initial one in the shaft (main body) of the columella and then a second site at the base of the footplate (Wood et al., 2010). The relatively late differentiation of the footplate base in the chick is similar to our studies in the mouse, which have shown that the mesodermal part of the footplate develops later than the neural crest part.

Interestingly the extent of the mesodermal tissue in the footplate is rather limited, particularly when comparing it to the amount of columella tissue lost after ablation of the otic capsule (Zou et al., 2012). It would therefore appear likely that the

otic capsule plays two roles, one in providing the cells for the footplate, and another involved in inducing the correct shape of the neural crest derived part of the ossicle.

Positioning of the annular ligament

As with development of the cranial coronal suture at the interface between the neural crest-derived frontal bones and the mesoderm-derived parietal bones, it might be expected that the annular ligament would be found at the interface of neural crest and mesodermal cells, with the two cells types signaling differentially to induce annular ligament formation. However we have shown this ligament forms within mesoderm-derived tissue, between the mesoderm derived otic capsule and the mesoderm-derived part of the stapedial footplate. Therefore, annular ligament formation is in fact more comparable to the development of the sagittal suture rather than the coronal suture. The development of this suture is correlated to the underlying junction of the two cerebral hemispheres. The oval window region does not form chondrocytes, suggesting that either it is intrinsically unable to do so, or inhibited from doing so by the surrounding tissue. In cases of otosclerosis the oval window does ossify indicating that it does have the potential to form skeletal tissue. An inhibition of chondrogenesis therefore appears likely. One candidate is the Bmp antagonist *Noggin*, which is expressed around the stapedial footplate (Hwang & Wu, 2008). It is also possible that the cells in this oval window region may have a role in the development of the neighbouring mesodermal cells in the otic capsule and footplate. Whatever controls annular ligament formation would be responsible for stapes fixation when the ligament does not form and therefore this is an important area to pursue.

The neural crest part of the stapes is needed for formation of the mesodermal footplate and for normal oval window development

From data from human development, the stapes appears to play an important role in the induction of the oval window. Using CT scans, or following middle ear surgery it has been seen that in the majority of cases when the oval window is absent the stapedial footplate is absent, reduced or displaced (Everberg, 1968; Jahrsdoerfer, 1977; Booth et al., 2000; Zeifer et al., 2000; Park et al., 2009). In keeping with this, the *Hoxa2* mutant mouse lacks a stapes and appears to have no oval window

(Gendron-Maguire et al., 1993; Rijli et al., 1993; Kanzler et al., 1998). However, as the stapedial footplate is a composite structure made of both neural crest and mesodermal derived cells either component might play the role of inducer. Using a mouse with loss of neural crest derived tissue, we have shown that the neural crest cartilage is needed for the formation of the mesodermally derived stapedial footplate, and in the absence of neural crest cartilage the oval window does not form correctly. In the *Wnt1creDicer* mice, neural crest cells are able to migrate and are found within the same locations as in control littermates at E11 (Zehir et al, 2010), but cells do not differentiate into cartilage but undergo apoptosis (Huang et al., 2010; Zehir et al., 2010). It is possible therefore that the migrating neural crest cells at E11 are able to signal to the otic capsule, positioning the oval window prior to stapes differentiation. However, our results are similar to those described in the turtle after complete ablation of the neural crest, where the columella is lost and the oval window has the appearance of an irregular fissure (Toerien, 1965), indicating that neural crest is not necessary for oval window initiation. A similar initiation but abnormal development of the oval window was observed after addition of retinoic acid to mouse embryos. Retinoic acid is thought to affect the migration of the neural crest, and in cases where the ossicles failed to form the oval window was still present, as a small opening in the otic capsule (Mallo, 1997). A small oval window and loss of the stapes is also seen in Noggin transgenics, which drive over-expression of Noggin in *Hoxa2* expressing tissue (Kanzler et al., 2000). The position of the oval window therefore appears not to be dependent on the stapes, or neural crest derived tissue. The complete loss of the oval window in *Hoxa2* mutants may be linked to additional defects in the development of the otic capsule (Gendron-Maguire et al., 1993; Rijli et al., 1993). Given the mouse studies it would be predicted that some form of an oval window would form in patients where the stapes is defective. In keeping with this, a thinning and dimpling in the region of the normal oval window have been reported by microCT in patients with stapes defects indicating that, similar to the *Wnt1creDicer* knockout mice, patients develop a remnant of the oval window in the absence of the stapes (Zeifer et al., 2000). The signals responsible for the initial position of the oval window are therefore independent of interactions with the stapes. The correct development of the oval window after initiation, however, is dependent on the presence of the neural crest part of the stapes. If the stapes does not provide the signal for oval window initiation, induction might be linked to signals from the underlying

otic epithelium. The vestibule of the inner ear is seen close to the oval window at early stages of ear development (see Figure 3A) and is a potential source of signals allowing the correct alignment of the inner and middle ears.

In our lineage studies the oval window part of the otic capsule was shown to have a mesodermal origin, however, we confirmed that some other parts of the otic capsule are indeed derived from neural crest (Toerien, 1963; Le Lièvre, 1978; Noden, 1983; O'Gorman, 2005). The crest-derived region is rather small and appears in wholemount to be the same as that region shown by O'Gorman (2005) to be derived from the second pharyngeal arch. The small positive region appears continuous with the developing styloid process (Reichert's cartilage), which has been shown to be of second arch origin. The first and more posterior arch crest streams therefore do not appear to contribute to otic capsule cartilage. This region of the capsule attaches to the squamosal bone and forms part of the roof of the middle ear cavity and is lost in the *Wnt1creDicer* mutants. A neural crest origin for this part of the otic capsule is therefore highly conserved, having been observed in birds, mammals and amphibians, and may play a role in the coordinated development of this part of the otic capsule with the surrounding neural crest derived jaw and ear bones.

Experimental Procedures

Animals

Heterozygous *Wnt1cre* (Danielian et al., 1998) and *Mesp1cre* (Saga et al., 1999) males were mated overnight with homozygous R26R (Soriano, 1999) females. Noon after the detection of the vaginal plug was considered as embryonic day (E) 0.5. All animals were killed using schedule 1 method as approved by the Home Office and King's College London and fixed in 4% PFA for 45 minutes for X-Gal, or 2 hours for Trichrome staining. Positive embryos were selected using a quick X-Gal staining procedure on the embryonic trunks (20 minutes in X-Gal solution at 37^{o}C). *Dicer$^{fl/fl}$*, mice were produced as described by Harfe (2005). *Wnt1cre/Dicer$^{fl/fl}$* mice were bred on a CD1 genetic background. 10 *Wnt1cre*/ R26R, 2 *Mesp1cre*/ R26R and 6 *Wnt1cre*/ Dicer$^{fl/fl}$ mice were analysed. As expected no variation was observed between LacZ stained samples.

Trichrome staining on paraffin sections

Following fixation, postnatal samples were decalcified in EDTA for up to 3 weeks then washed in distilled water, before washing in PBS. Embryonic samples were washed in PBS. Samples (n=9) were then dehydrated through a methanol series, cleared in tetrahydronapthalene and embedded in paraffin wax. 10µm frontal sections were mounted on Superfrost Plus Slides, dewaxed in Histoclear, rehydrated through IMS, stained with 1% Alcian Blue in 3% acetic acid pH2.5, Ehrlich's Haematoxylin and 0.5% Sirius Red in saturated Picric acid then mounted in DPX.

Wholemount LacZ staining of stapes and otic capsule

Following fixation, embryonic or postnatal mice (n=11) were washed in PBS and the otic capsules were dissected. These were washed twice in PBS/2mM $MgCl_2$ for 10 minutes at room temperature, followed by 5 minutes in Solution B (PBS/0.1% Deoxycholic acid/0.2% NP-40/2mM $MgCl_2$), then 3-5 hours in Solution C (Solution B/ 2.5mM $K_3Fe(CN)_6$/ 2.5mM $K_4Fe(CN)_6$/1mg/ml X-Gal) at 37^{o}C. The otic capsules were then washed and the stapes' dissected in some cases before imaging on the Leica Dissecting microscope.

Wholemount Alcian Blue staining of stapes

Following fixation, postnatal mice were washed in PBS and the stapes dissected. Isolated stapes were dehydrated in 95% ethanol for 1 hour and stained with 0.3% Alcian Blue in 70% ethanol, acetic acid and 70% ethanol at a ratio of 1:1:18 overnight at 37^{o}C. Finally the dissected stapes were rinsed in distilled water and imaged as above.

LacZ staining and immunohisotchemistry on cryosections

Embryonic and postnatal mouse heads were cryo-embedded and sectioned frontally at a thickness of 20µm. Sections were stained in X-Gal, following the same procedure for wholemount X-Gal staining, for 4-6 hours. These were then incubated with rabbit polyclonal Sox9 (Millipore; 1:100) overnight. The sections were then washed and incubated in goat anti-rabbit 568 (1:500; Invitrogen) before mounting in SlowFade Gold antifade reagent with DAPI (Invitrogen). Slides were imaged under a Zeiss Akioskop 2 plus microscope. LacZ and immuno-double stained sections were blended

in Adobe Photoshop CS4 using the blending option Luminosity, with the fluorescent image over the brightfield image.

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Figures Legends

Figure 1. The development of the stapes

(A) Schematic illustrating the developing stapes and surrounding structures. (B) Schematic illustrating the structure of the stapes and its position in the oval window. (C-F) Trichrome staining of frontal sections of mouse middle ear showing the stapes at E14.5 (C), E16.5 (D), E18.5 (E) and P6 (F). At E14.5 (C), the footplate can be seen within the otic capsule. At E16.5 (D) and E18.5 (E), the position of the annular ligament can be seen (black arrow heads). The base of the footplate showing delayed development compared to the body of the stapes (black arrows). At P6 (F), the annular ligament can be clearly determined (arrow heads). The footplate of the stapes is on the right and head of the stapes on the left. st- stapes; oc- otic capsule; fn – facial nerve; sa – stapedial artery. Scale bar: 100μm.

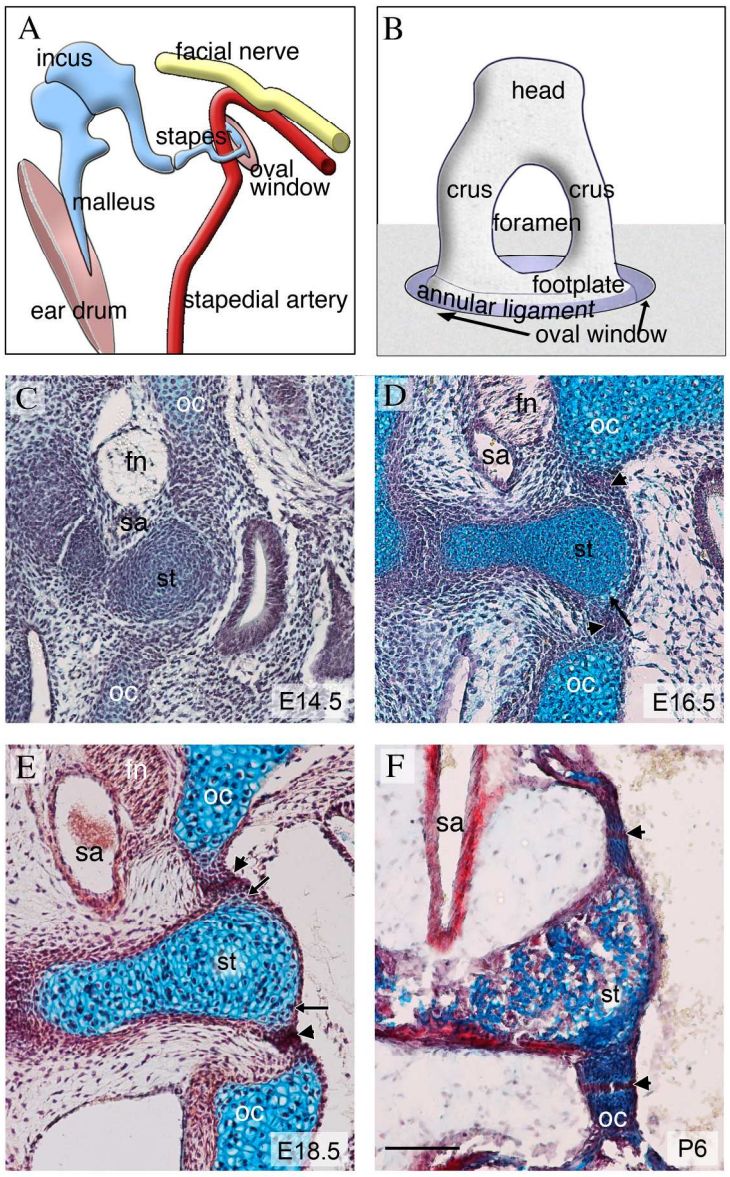
Figure 2. The stapes is of dual origin

(A) Alcian Blue staining of a stapes (P6) showing it is fully cartilaginous at this stage. (B) Wholemount X-Gal staining of a *Wnt1cre*/R26R stapes, showing part of the footplate is unstained (black arrow) at P10. (C) Wholemount X-Gal stained *Mesp1cre*/R26R stapes, showing part of the footplate stained blue (black arrow) at P10. (D) Schematic illustrating the stapes in the oval window and the orientation of sectioning in E and F. (E, F) Frontal section through a stapes following X-Gal and eosin staining of a *Wnt1cre*/R26R (E) and *Mesp1cre*/R26R (F) showing LacZ-negative and LacZ-positive cells in the stapedial footplate. The annular ligament (black arrow head) and oval window membrane (white arrow) are negative in the *Wnt1cre*/R26R section and positive in the *Mesp1cre*/R26R section. (G) Lateral view of the left otic capsule of an E18.5 *Wnt1cre*/R26R mouse following X-Gal staining. LacZ positive tissue is indicated by the arrow in the pars canalicularis of the otic capsule. Reichert's cartilage (styloid process) can also be seen labeled blue. The dashed line indicates the plane of section shown in G. (G) Frontal section through a region of otic capsule following X-Gal and eosin staining of a *Wnt1cre*/R26R. The arrow indicates the LacZ-positive otic capsule cartilage at a distance from the oval window. The otic capsule is outlined. co- cochlear; fn- facial nerve; i- incus; ie- inner ear; ma- malleus; me- midde ear; oc- otic capsule; oc-pc- otic capsule- pars

canalicularis; r, Reichet's cartilage; sa- stapedial artery; st- stapes. Scale bar: A-C, 100μm; E-F, 50μm; G, 300μm; H, 100 μm.

Figure 3. Development of the neural crest-derived stapes in the otic capsule
(A, B) X-Gal staining of *Wnt1cre*/R26R frontal sections showing the neural crest derived cells at E12.5 (arrow) (A) and E15.5 (B). (A', B') Immunostaining against Sox9 showing developing chondrocytes and the inner ear epithelium at E12.5 (A') and E15.5 [B'). (A'') Adobe Photoshop image blend of A and A' showing *Wnt1cre*-positive and Sox9-positive cells (blue) and Sox9-positive only cells (white) at E12.5. (B'') Photoshop image blend of B and B' showing *Wnt1cre*-positive and Sox9-positive cells (blue) and Sox9-positive only cells (white) at E15.5. The stapes footplate is composed of neural crest-derived (arrow) and non-neural crest-derived cells (arrow head). oc- otic capsule; ve- vestibule of inner ear; s- stapes. Scale bar: 50μm.

Figure 4. Neural crest cartilage is needed for normal footplate and oval window development
(A, B, E-F) Trichrome staining of E18.5 frontal sections. (C-D) Schematics. (G-H) H+E staining of E18.5 frontal sections. (A) Wildtype section showing middle (arrows) and inner ear cartilages. (B) *Wnt1cre*/Dicer showing inner ear cartilage and lack of middle ear cartilage. (C) Schematic illustrating the structure of the stapes within the oval window. The mesoderm-derived component of the stapes is coloured green (arrow), while the neural crest stapes is grey (arrow head). (D, D') Two possible outcomes of loss of the neural crest part of the stapes. (E-F) Section through the same position as C. (E) A wildtype mouse through the stapes. (F) *Wnt1cre*/Dicer mutant showing lack of cartilage within the oval window (*). (G) Section through the otic capsule showing the cartilaginous projection from the pars canalicularis (arrow) in a wildtype mouse. (H) A *Wnt1cre*/Dicer section showing the same position with loss of the crest derived cartilage projection.
st – stapes; i – incus; oc – otic capsule.
Scale bar: A-B, 120 μm; E-F, 25 μm. G-H, 50 μm.



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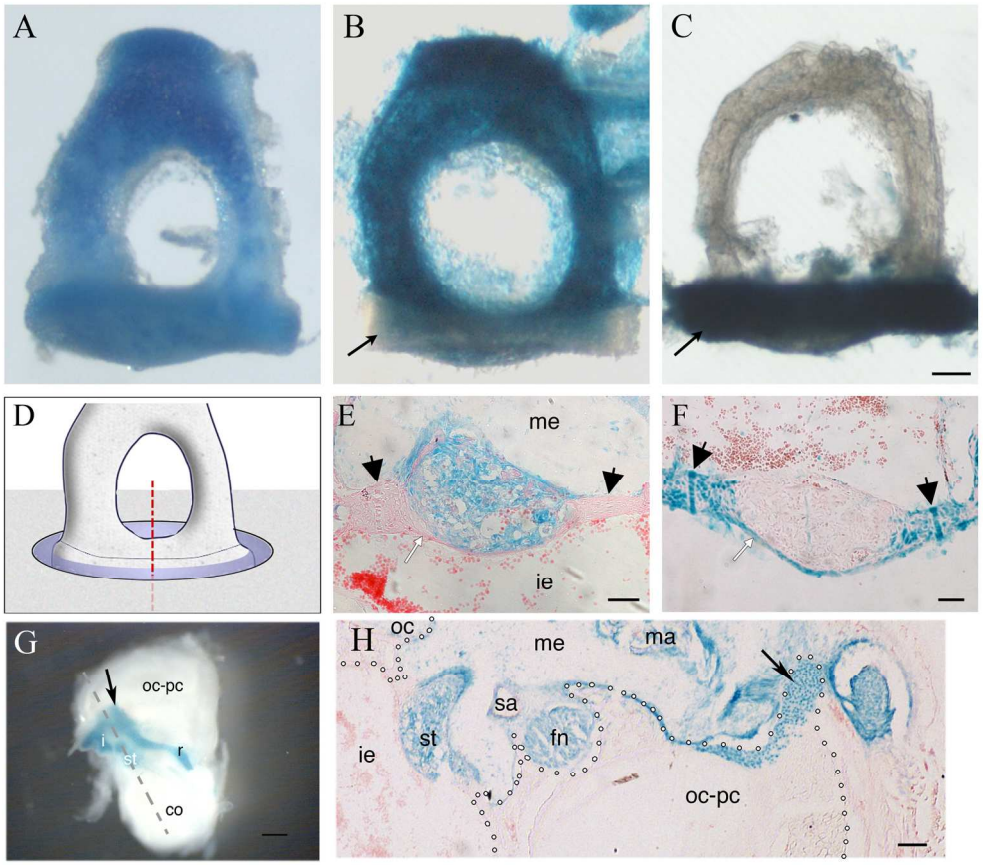

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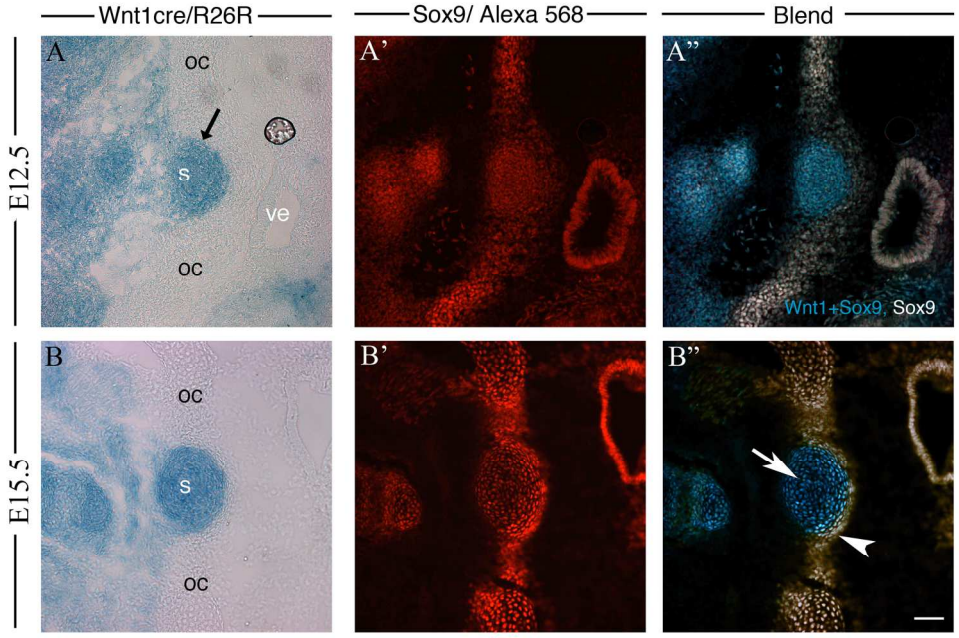

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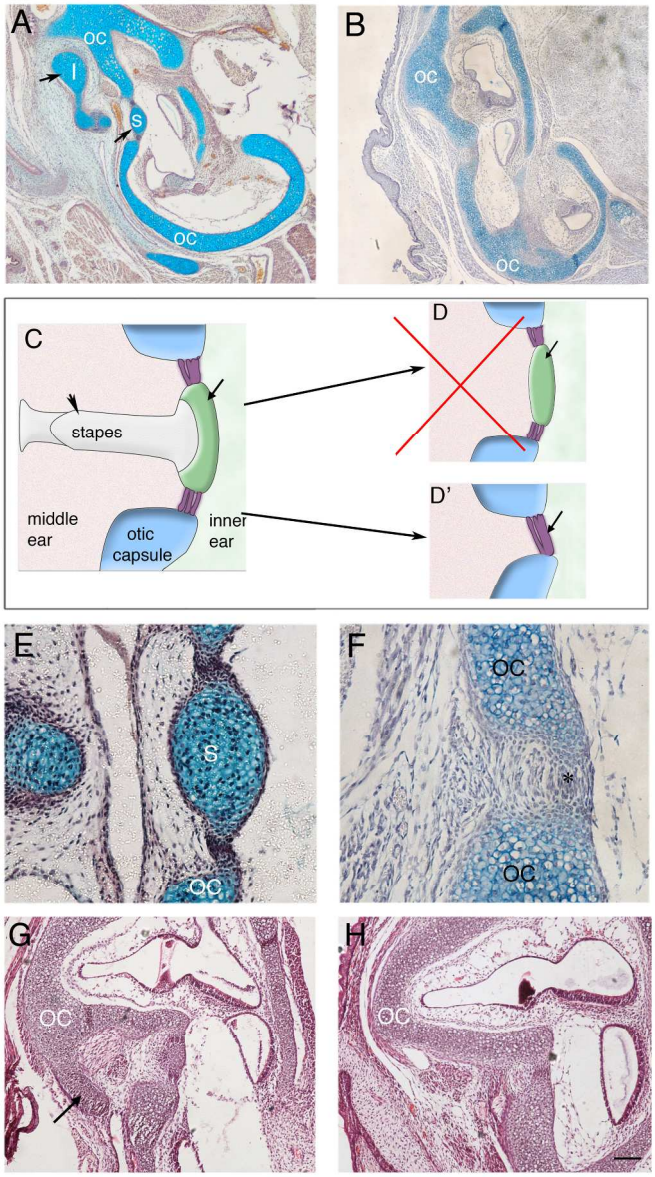

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